

SUPPLEMENTAL MATERIAL**CONTRIBUTION OF IMPAIRED MYOCARDIAL INSULIN SIGNALING TO MITOCHONDRIAL DYSFUNCTION AND OXIDATIVE STRESS IN THE HEART**

Sihem Boudina, Ph.D.¹, Heiko Bugger M.D.¹, Sandra Sena, Ph.D.¹, Brian T. O'Neill, MD, Ph.D.¹, Vlad G. Zaha, MD Ph.D.¹, Olesya Ilkun, MS.¹, Jordan J. Wright, BS.¹, Pradip K. Mazumder, DVM Ph.D.¹, Eric Palfreyman, MD.¹, Timothy J. Tidwell¹, Heather Theobald, BS.¹, Oleh Khalimonchuk, Ph.D.², Benjamin Wayment, BS³, Xiaoming Sheng Ph.D.⁴, Kenneth J. Rodnick, Ph.D.⁵, Ryan Centini BS⁶, Dong Chen Ph.D⁶, Sheldon E. Litwin, MD.³, Bart E. Weimer Ph.D.⁶, E. Dale Abel, MB.BS., D.Phil.^{1,2}

SUPPLEMENTAL METHODS**Composition of animal chow**

Animals were fed standard mouse chow Harland Teklad Diet 8656 that contained 24.5% protein (Soy based), 65% Carbohydrate (Corn and soybean meal), 4.4% fat (Soybean oil), 3.4% fiber and supplemented with vitamins and minerals. This diet provided 3.8 Kcal/g of gross energy.

Mitochondrial Isolation and Determination of Oxygen Consumption

Hearts were diced in STE medium [250 mmol/l sucrose, 5 mmol/l Tris/HCl (pH 7.4) and 2 mmol/l EGTA], digested on ice for 4 min in STE medium [to which was added 0.5% (w/v) BSA, 5 mmol/l MgCl₂, 1 mmol/l ATP and 2.5 units/ml protease (type VIII from *Bacillus licheniformis*)] and homogenized three times in a Polytron homogenizer for 3 seconds. The homogenate was then centrifuged at 8,000X g for 10 min at 4 °C. The

pellets were re-suspended in STE buffer and centrifuged at 700g for 10 min at 4 °C. Finally, the supernatant is centrifuged at 8,000X g for 10 min at 4 °C to pellet the mitochondria. The protein concentration of mitochondrial suspensions was determined using a Micro BCA reagent (Pierce) with BSA as a standard. Respirations on isolated mitochondria (0.4 mg/ml) were measured at 25 °C in respiration buffer [120 mmol/l KCl, 5 mmol/l KH₂PO₄, 1 mmol/l EGTA, 3 mmol/l HEPES, 1 mg/ml BSA with either 20 μmol/l palmitoyl-carnitine with 2 mmol/l malate, or with 10 mmol/l pyruvate and 5 mmol/l malate, pH 7.2].

Blue Native Gel Electrophoresis

Mitochondrial membranes (75 μg) were solubilized using 1% dodecylmaltoside final concentration, incubated for 20 min on ice and centrifuged (20,000X g, 10 min, 4°C). Supernatants were mixed with sample buffer (5% Coomassie brilliant blue G250, 0.5 mole 6-aminocaproic acid, pH 7.0) and loaded on a 6–13% gradient polyacrylamide gel. After electrophoresis, gels were rinsed briefly with water, and horizontal gel strips were cut containing complex I, complex IV, or complex V. Gel strips were equilibrated in respective staining solution without staining reagents for 10 min, and then incubated in 10ml staining solution as described before ¹. In brief, Complex I solution contained 0.1 mg/mL NADH, 2 mmol/l Tris/HCl, and 2.5 mg/mL Nitro Blue tetrazolium chloride, pH 7.4. Complex IV solution contained 50 mmol/l phosphate buffer pH 7.4, 1 mg/ml 3,3'-diaminobenzidine, 10 μL catalase (20 mg/mL), 1 mg/ml cytochrome c, and 220 mmol/l sucrose. Complex V solution contained 34 mmol/l Tris, 270 mmol/l glycine, 14 mmol/l

MgSO₄, 0.2% lead acetate, and 8 mmol/l ATP, pH 7.8. Gel strips were incubated at room temperature (complex I) or 30°C (complex IV and V) for 10 min. Gels were then destained and fixed in 45% (v/v) methanol and 10% (v/v) acetic acid. Signal intensity was quantified using Image J software.

Mitochondrial Proteomic Analysis

Protein in-solution Tryptic Digestion: 5µl of 0.2% RapidGest (Waters, Manchester, UK) was added to 20µg of membrane protein sample in 15µl H₂O. The mixed solution was heated at 80°C for 20 minutes. The protein mixtures were then tryptically digested according to a modified digestion method in the Waters Protein Expression System Manual (Waters, 2006). After adding NH₄HCO₃ and treating with dithiothreitol (DTT) and iodoacetamide (IAA), 4 µl of 0.11 µg/µl Trypsin in 25 mM NH₄HCO₃ was added to the protein sample. The sample was then incubated at 37°C overnight and then with 1% Formic Acid for 30 minutes at 37°C, and then centrifuged at 10,000 RPM for 10 minutes. The supernatant is then analyzed by LC-MS/MS.

Expression Analysis by Mass Spectrometry: Digested protein samples (3 µl each) were introduced into a Symmetry® C18 trapping column (180 µM x 20 mm) by NanoACQUITY Sample Manager (Waters, Manchester, UK) and washed by H₂O for 2 minutes at 10 ml/min. The peptides were then eluted from the Trapping column over a 100 µm x100 mm BEH 130 C18 column with a 140 min gradient (1-4% B for 0.1 min, 4-25% B for 89.9 min, 25-35% B for 5 min, 35-85% B for 2 min, 85% B for 13 min, 85-95% B for 8 min, 95-1% B for 2 min and 1% B for 20 min) at 0.8µL/min flow rate using an

NanoACQUITY UPLC (Waters, Manchester, UK). For this system, solvent A is composed of 99.9% H₂O, 0.1% formic acid. Solvent B is composed of 99.9% acetonitrile and 0.1% formic acid. The mass spectrometry (MS) was set to a parallel fragmentation mode (MSE) with the scan times of 1.0 second. The low fragmentation energy was 5 volts and the high fragmentation ranged from 17 to 45 volts. (GLU1)-Fibrinopeptide B is used as an external calibration standard with LockSpray. Enolase is used as the spiked control. Waters ProteinLynx Global SERVER Version 2.3 was used to analyze the ms dataset.

LC/MS/MS data statistical analysis: The algorithms and statistical method used in the Waters Protein Expression System are published ². The key algorithm in Waters Protein Expression System is the clustering algorithm which chemically clusters peptide components by mass and retention time for all injected samples and performs binary comparisons for each of the experimental condition to generate an average normalized intensity ratio for all matched AMRT (Accurate Mass, Retention Time) components. A statistical Student's *t*-test method was used for each binary comparison.

Insulin signaling: Total proteins were extracted from whole hearts of 8 week-old mice that were perfused in the working mode with Henseleit buffer containing (in millimoles) 118.5 NaCl, 25 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂, 0.5 EDTA, and 5 glucose, gassed with 95% O₂-5% CO₂ and supplemented with 0.4 mM palmitate bound to 3% BSA in the presence or absence of 1 nM insulin. Hearts were frozen in liquid nitrogen and homogenized with a Polytron in Lysis Buffer (Hepes, 50 mM, pH7.5; NaCl, 150 mM; glycerol, 10%; sodium pyrophosphate, 10 mM; sodium fluoride, 100 mM;

EGTA, 1 mM; Triton X-100, 1%; protease and phosphatase inhibitor cocktail from Roche and Sigma, respectively). Samples were then incubated for 1 hour on ice and then centrifuged for 10 minutes at 14,000 g in a micro-centrifuge at 4°C. The supernatant was collected, aliquoted and stored at -80°C until use. Protein concentration was measured using Micro BCA reagent (Pierce).

Western blot analysis.

For insulin signaling, protein extracts were resolved by SDS-PAGE and electrotransferred onto PVDF membranes (Millipore Corp., Billerica, MA). Membranes were probed with the appropriate primary antibody at a dilution of 1/1000. The following antibodies were used: phospho-Akt-Ser 473 (Cell Signaling Danvers, MA), total Akt1/2 (Cell Signaling), IGF1R (Santa Cruz, Santa Cruz, CA), phospho-GSK3 β -Ser9 (Cell Signaling), total GSK3 β (Santa Cruz). Other antibodies used were: rabbit anti-catalase antibody (Novus Biologicals, Littleton, CO), mouse anti-MnSOD antibody (BD Biosciences, San Jose, CA), a mouse polyclonal antibody against aconitase (kind gift of Dr. Elizabeth Leibold, University of Utah), mouse anti- Cytochrome Oxidase (Complex IV) Subunit 4 (Molecular Probes, Eugene, OR) and Mfn2 (Sigma, Saint Louis, MO). To control for loading, mouse anti- α tubulin antibody (Sigma, Saint Louis, MO) was used. Protein detection was carried out with the appropriate horseradish peroxidase-conjugated secondary antibody and ECL or ECL Plus detection systems (Amersham Biosciences, Piscataway, NJ). Blots were scanned and analyzed by computerized laser densitometry using the Image J software and results were normalized against respective loading control. For pyruvate dehydrogenase E1 α (PDHE1 α) bots: Mouse

polyclonal to PDHE1 α subunit (Abcam Inc. Cambridge, MA) and the anti-Pyruvate Dehydrogenase pSer²⁹³ Rabbit pAb (Calbiochem, San Diego, CA) were used. Alexa fluor anti-Rabbit 680 (Invitrogen, Carlsbad, CA) and Mouse 800 (VWR International, West Chester, PA) were used as secondary antibodies and fluorescence quantified using the LI-COR Odyssey imager (Lincoln, NE).

Mitochondrial enzyme activities

Total carnitine palmitoyl-transferase (CPT) (CPT 1 and CPT 2) activity was measured in isolated mitochondria from freshly excised non-perfused hearts as previously described³. Citrate synthase (CS) and 3-hydroxyacyl-CoA dehydrogenase (HADH) enzyme activities were assessed in frozen heart tissue (~10 mg) as previously described³. Aconitase activity was measured in mitochondrial fractions as described before⁴.

SUPPLEMENTAL TABLES

Supplemental Table S1. Primer sequences of mouse genes used for quantification of mRNA levels by real-time PCR.

Gene Name	Sequence of forward and reverse primers (5'-3')	GeneBank reference
Medium chain acetyl-Coenzyme A dehydrogenase (ACADM)	ACTGACGCCGTTCAGATTTCCTTAGTTACACGAGGGTGATG	NM_007382
Carnitine palmitoyltransferase 1b – muscle (CPT1B)	TGCCTTACATCGTCTCCAAAGACCCCGTAGCCATCATC	NM_009948
Carnitine palmitoyltransferase 2 (CPT2)	CCAGCTGACCAAAGAAGCA GCAGCCTATCCAGTCATCGT	NM_009949
Pyruvate dehydrogenase kinase - isoenzyme 4 (PDK4)	GCTTGCCAATTCTCGTCTC CTTCTCCTTCGCCAGGTTCT	NM_013743
Peroxisome proliferator activated receptor alpha (PPARA)	GAGAATCCACGAAGCCTACC AATCGGACCTCTGCCTCTTT	NM_011144
Peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC1A)	GTAAATCTGCGGGATGATGG AGCAGGGTCAAAATCGTCTG	NM_008904
Peroxisome proliferator activated receptor gamma co-activator 1 beta (PGC1B)	TGAGGTGTTCGGTGAGATTG CCATAGCTCAGGTGGAAGGA	NM_133249
Estrogen related receptor alpha (ESRRA)	GGAGGACGGCAGAAGTACAA CAGGTTCAACAACCAGCAGA	NM_007953
Transcription factor A – mitochondrial (TFAM)	CAAAAAGACCTCGTTCAGCA CTTCAGCCATCTGCTCTTCC	NM_009360
Nuclear respiratory factor 1 (NRF1)	CTTCAGAACTGCCAACCA GCTTCTGCCAGTGATGCTAC	NM_010938
Nuclear respiratory factor 2 (NFR2)	AGTCTTCACTGCCCTCATC TCTGTCAGTGTGGCTTCTGG	NM_010902

Uncoupling protein 2 (UCP2)	TCTCCTGAAAGCCAACCTCA CTACGTTCCAGGATCCCAAG	NM_011671
Uncoupling protein 3 (UCP3)	TGCTGAGATGGTGACCTACGA CCAAAGGCAGAGACAAAGTGA	NM_009464
Fatty acid translocase (CD36)	TCCTTGTAGGAGATGGTGTTC CCATTGGTGATGAAAAAGCA	NM_007643
Fatty acid binding protein 3 - muscle and heart (FABP)	GACGGGAAACTCATCCTGAC TCTCCAGAAAAATCCCAACC	NM_010174
Mitochondrial creatine kinase 2 (CKMT2)	AGAACTGCGGCTCCAAAAG CACTTCCTGCCAAACTGAGG	AK009042
Mitochondrial acyl-CoA thioesterase 1 (MTE1)	GACCTCCCCAAGAGCATAGA TCCTTGTAGGAGATGGTGTTC	NM_134188
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (Ndufa9)	ATCCCTTACCCTTGGCCACT CCGTAGCACCTCAATGGACT	NM_025358
Ubiquinol-cytochrome c reductase core protein 1 (Uqcrc1)	TGCCAGAGTTCCAGACCTT CCAAATGAGACACCAAAGCA	NM_025407
Cyclophilin A (CPHN)	AGCACTGGAGAGAAAGGATTG G TCTTCTTGCTGGTCTGCCATT	NM_008907
Pyruvate dehydrogenase E1 α 1 (PDHA1)	GGGACGTCTGTTGAGAGAGC TGTGTCCATGGTAGCGGTAA	NM_008810

Primer pairs were designed based on GenBank reference sequences. We used the WWW interface Primer 3 (http://frodo.wi.mit.edu/cgi_bin/primer3/primer3_www.cgi) with default settings. To avoid non specific amplifications, primer sequences were blasted against mouse genes. Dissociation curves were analyzed for all primer-pairs to ensure single product amplification.

Supplemental Table S2A. Proteomic Analysis of all Mitochondrial Membrane Proteins from CIRKO Mice.

Accession	OK	Description	Score	Unique	Matrix -WT- 20070 913:M atrix- TG- 20070 913_ Ratio	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) Ratio	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) StdDe v	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) P	Matrix -WT- 20070 913:M atrix- TG- 20070 913_R el	Matrix- TG- 20070 913_R el	Matrix- TG- 20070 913_R el Profile SD	Matrix- WT- 20070 913_R el Profile SD	Matrix- WT- 20070 913_R el Profile SD
NP_080448.1	2	2,4-dienoyl CoA reductase 1, mitochondrial [Mus musculus]	358.88		1.16	0.15	0.12	0.99	0.92	0.06	1.08	0.06	
NP_077150.1	2	3-oxoacid CoA transferase 1 [Mus musculus] acetyl-Coenzyme A acetyltransferase 1 precursor [Mus	371.67		0.81	-0.21	0.19	0.01	1.11	0.09	0.89	0.09	
NP_659033.1	2	musculus] acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-	648.11		1.05	0.05	0.1	0.83	0.97	0.05	1.03	0.05	
NP_803421.1	2	oxoacyl-Coenzyme A thiolase) [Mus musculus] acetyl-Coenzyme A dehydrogenase, long-chain [Mus	806.71		1.2	0.18	0.08	1	0.91	0.04	1.09	0.04	
NP_031407.2	2	musculus] acetyl-Coenzyme A dehydrogenase, medium chain [Mus	730.49		0.88	-0.13	0.08	0.01	1.06	0.04	0.94	0.04	
NP_031408.1	2	musculus] acetyl-Coenzyme A dehydrogenase, medium chain [Mus	474.11		1.08	0.08	0.14	0.88	0.96	0.07	1.04	0.07	
NP_542364.1	2	aconitase 2, mitochondrial [Mus musculus] acyl-CoA synthetase long-chain family member 1 [Mus	1275.5	3	1	0	0.06	0.52	1	0.03	1	0.03	
NP_032007.2	2	musculus] acyl-CoA synthetase long-chain family member 5 [Mus	488.25		1.15	Memb rane-WT- 2007091	Memb rane-WT- 20070	Memb rane-WT- 20070	Memb rane-WT- 20070	Memb rane-WT- 20070			
NP_082252.1	2	musculus] acyl-CoA dehydrogenase, very long chain [Mus	234.02	3	1.25	0.22	0.07	1	0.89	0.03	1.11	0.03	
NP_059062.1	2	musculus] ALBU_BOVIN (P02769) Serum albumin precursor (Allergen	1665.1		0.9	-0.1	0.07	0	1.05	0.04	0.95	0.04	
NP_000101.0	2	Bos d 6) (BSA)	1518.2		0.92	-0.08	0.1	0.06	1.04	0.05	0.96	0.05	
NP_904332.1	2	ATP synthase F0 subunit 8 [Mus musculus] ATP synthase, H ⁺ transporting mitochondrial F1 complex,	168.47		1.01	0.01	0.03	0.78	0.99	0.02	1.01	0.02	
NP_058054.2	2	beta subunit [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F0 complex,	3570.9	5	0.98	-0.02	0.09	0.34	1.01	0.04	0.99	0.04	
NP_033855.2	2	subunit b, isoform 1 [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F0 complex,	566.83		0.97	-0.03	0.17	0.38	1.01	0.09	0.99	0.09	
NP_082138.1	2	subunit d [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F0 complex,	1064.7	4	1.03	0.03	0.06	0.81	0.99	0.03	1.01	0.03	
NP_058035.1	2	subunit F [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F0 complex,	270.62		1.02	0.02	0.13	0.63	0.99	0.07	1.01	0.07	
NP_065607.1	2	subunit f, isoform 2 [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F1 complex,	99.12		0.97	-0.03	0.17	0.38	1.01	0.09	0.99	0.09	
NP_031531.1	2	alpha subunit, isoform 1 [Mus musculus]	3436.1	1	1.04	0.04	0.03	0.95	0.98	0.02	1.02	0.02	

NP_079589.1	2	ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit precursor [Mus musculus]	290.22	0.93	-0.07	0.1	0.05	1.03	0.05	0.97	0.05
NP_080259.1	2	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit [Mus musculus]	115.91	1.01	0.01	0.12	0.6	0.99	0.06	1.01	0.06
NP_065640.1	2	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma subunit [Mus musculus]	442.44	0.92	-0.08	0.09	0.05	1.04	0.04	0.96	0.04
NP_613063.1	2	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit [Mus musculus]	598	0.98	-0.02	0.06	0.24	1.01	0.03	0.99	0.03
NP_031557.1	2	B-cell receptor-associated protein 37 [Mus musculus]	291.71	0.95	-0.05	0.22	0.32	1.02	0.11	0.98	0.11
NP_031786.2	2	carnitine acetyltransferase [Mus musculus]	383.32	1.27	0.24	0.1	1	0.88	0.05	1.12	0.05
NP_034079.1	2	carnitine palmitoyltransferase 2 [Mus musculus]	539.29	1.46	0.38	0.16	1	0.81	0.08	1.19	0.08
NP_080720.1	2	citrate synthase [Mus musculus]	520.02	0.93	-0.07	0.1	0.08	1.04	0.05	0.96	0.05
NP_082221.1	2	citrate synthase-like protein [Mus musculus]	349.23	0.89	-0.12	0.13	0.04	1.06	0.06	0.94	0.06
NP_034027.1	2	creatine kinase, mitochondrial 1, ubiquitous [Mus musculus]	189.17	1.19	0.17	0.15	0.98	0.91	0.08	1.09	0.08
NP_940807.1	2	creatine kinase, mitochondrial 2 [Mus musculus]	1125.8	1.31	0.27	0.07	1	0.87	0.03	1.13	0.03
NP_904331.1	2	cytochrome c oxidase subunit II [Mus musculus]	623.63	1.06	0.06	0.08	0.93	0.97	0.04	1.03	0.04
NP_034071.1	2	cytochrome c oxidase subunit IV isoform 1 [Mus musculus]	540.31	0.92	-0.08	0.04	0	1.04	0.02	0.96	0.02
NP_031773.1	2	cytochrome c oxidase, subunit Va [Mus musculus]	441.94	1	0	0.07	0.56	1	0.04	1	0.04
NP_034072.1	2	cytochrome c oxidase, subunit Vb [Mus musculus]	238.62	1.05	0.05	0.1	0.86	0.97	0.05	1.03	0.05
		cytochrome c oxidase, subunit VIb polypeptide 1 [Mus									
NP_079904.1	2	musculus]	357.74	0.95	-0.05	0.12	0.24	1.03	0.06	0.97	0.06
NP_444301.1	2	cytochrome c oxidase, subunit VIc [Mus musculus]	239.43	1	0	0.12	0.52	1	0.06	1	0.06
NP_034074.1	2	cytochrome c oxidase, subunit VIIa 1 [Mus musculus]	253.05	1	0	0.11	0.51	1	0.06	1	0.06
NP_031834.1	2	cytochrome c, somatic [Mus musculus]	318.58	1.15	0.14	0.13	0.98	0.93	0.06	1.07	0.06
NP_079843.1	2	cytochrome c-1 [Mus musculus]	351.45	1.15	0.14	0.09	1	0.93	0.04	1.07	0.04
NP_031887.2	2	dihydrolipoamide dehydrogenase [Mus musculus]	401.97	0.95	-0.05	0.16	0.2	1.02	0.08	0.98	0.08
NP_663589.2	2	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) [Mus musculus]	617.75	0.84	-0.17	0.14	0.01	1.08	0.07	0.92	0.07
NP_084501.1	2	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) [Mus musculus]	420.2	0.84	-0.18	0.1	0	1.09	0.05	0.91	0.05
NP_034153.2	2	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase) [Mus musculus]	304.45	1.01	0.01	0.17	0.5	0.99	0.09	1.01	0.09
NP_663590.2	2	electron transferring flavoprotein, alpha polypeptide [Mus	564.68	1.03	0.03	0.09	0.71	0.98	0.05	1.02	0.05
NP_080971.2	2	musculus]	761.25	1.01	0.01	0.09	0.54	1	0.05	1	0.05
NP_080070.1	2	electron transferring flavoprotein, dehydrogenase [Mus	911.73	1.16	0.15	0.08	1	0.92	0.04	1.08	0.04
		ENO1_YEAST (P00924) Enolase 1 (EC 4.2.1.11) (2-phosphoglycerate dehydratase 1) (2-phospho-D-glycerate									
NP_000202.0	2	hydro-lyase 1)	541.65	0.91	-0.09	0.09	0	1.04	0.04	0.96	0.04
NP_444349.1	2	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial	418.4	0.99	-0.01	0.15	0.44	1.01	0.07	0.99	0.07
NP_034339.1	2	[Mus musculus]	347.48	1	0	0.18	0.54	1	0.09	1	0.09
NP_034455.1	2	fumarate hydratase 1 [Mus musculus]	1105.3								
NP_034611.1	2	glutamate oxaloacetate transaminase 2, mitochondrial [Mus	4	1.09	0.09	0.05	1	0.96	0.03	1.04	0.03
NP_058043.3	2	musculus]	520.72	0.79	-0.23	0.14	0.01	1.12	0.07	0.88	0.07
NP_849209.1	2	heat shock protein 9A [Mus musculus]	286.7	0.91	-0.09	0.2	0.25	1.04	0.1	0.96	0.1
		hydroxyacyl-Coenzyme A dehydrogenase type II [Mus	1630.5	1.11	0.1	0.06	0.99	0.95	0.03	1.05	0.03
		musculus]									
		hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-									

		Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit [Mus musculus]	8									
NP_663533.1	2	Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit [Mus musculus]	1098.0	1	1.32	0.28	0.06	1	0.86	0.03	1.14	0.03
NP_00102893												
7.1	2	hypothetical protein LOC237880 [Mus musculus]	380.08	0.86	-0.15	0.13	0	1.07	0.06	0.93	0.06	
NP_598768.1	2	hypothetical protein LOC52637 [Mus musculus]	100.29	1.27	0.24	0.36	0.92	0.88	0.16	1.12	0.16	
NP_932096.1	2	hypothetical protein LOC66152 [Mus musculus]	158.9	0.94	-0.06	0.18	0.31	1.03	0.09	0.97	0.09	
NP_080841.1	2	hypothetical protein LOC68117 [Mus musculus]	145.05	0.88	-0.13	0.26	0.22	1.07	0.13	0.93	0.13	
NP_083949.2	2	inner membrane protein, mitochondrial [Mus musculus] isocitrate dehydrogenase 2 (NADP+), mitochondrial [Mus	496.08	1	0	0.13	0.5	1	0.06	1	0.06	
NP_766599.1	2	musculus]	983.37	0.7	-0.35	0.09	0	1.18	0.04	0.82	0.04	
NP_083849.1	2	isocitrate dehydrogenase 3 (NAD+) alpha [Mus musculus]	519.36	0.99	-0.01	0.1	0.44	1.01	0.05	0.99	0.05	
NP_079628.1	2	low molecular mass ubiquinone-binding protein [Mus	247.08	0.97	-0.03	0.1	0.29	1.02	0.05	0.98	0.05	
NP_032643.2	2	musculus]	2239.4	0.97	0.07	0.05	0.99	0.97	0.03	1.03	0.03	
NP_077159.1	2	malate dehydrogenase 2, NAD (mitochondrial) [Mus	5	1.07	0.07	0.05	0.99	0.97	0.03	1.03	0.03	
NP_075801.1	2	musculus]	628.66	0.94	-0.06	0.13	0.2	1.03	0.06	0.97	0.06	
NP_035015.2	2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10	279.03	0.9	-0.1	0.11	0.1	1.05	0.06	0.95	0.06	
NP_035016.1	2	[Mus musculus]	167.09	0.87	-0.14	0.17	0.06	1.07	0.09	0.93	0.09	
NP_075691.1	2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13	475.85	0.94	-0.06	0.09	0.1	1.03	0.04	0.97	0.04	
NP_080979.1	2	[Mus musculus]	154.3	0.92	-0.08	0.21	0.23	1.04	0.11	0.96	0.11	
NP_079634.1	2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 7	340.26	0.92	-0.08	0.16	0.19	1.04	0.08	0.96	0.08	
NP_080960.1	2	(B14.5a) [Mus musculus]	806.81	0.89	-0.12	0.09	0.01	1.06	0.04	0.94	0.04	
NP_075661.1	2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 8	384.37	0.97	-0.03	0.12	0.28	1.01	0.06	0.99	0.06	
NP_077182.1	2	[Mus musculus]	228.67	0.9	-0.1	0.21	0.17	1.05	0.1	0.95	0.1	
NP_663493.1	2	NADH dehydrogenase (ubiquinone) 1, subcomplex	255.3	0.89	-0.12	0.15	0.06	1.06	0.07	0.94	0.07	
NP_694704.1	2	unknown, 2 [Mus musculus]	1608.6	0.9	-0.11	0.06	0	1.06	0.03	0.94	0.03	
NP_080964.1	2	NADH dehydrogenase (ubiquinone) Fe-S protein 1 [Mus	1	0.96	-0.04	0.13	0.28	1.02	0.06	0.98	0.06	
NP_035018.1	2	musculus]	537.15	0.94	-0.06	0.08	0.08	1.03	0.04	0.97	0.04	
NP_659119.2	2	NADH dehydrogenase (ubiquinone) Fe-S protein 6 [Mus	597.97	0.85	-0.16	0.15	0.02	1.08	0.07	0.92	0.07	
NP_598427.1	2	musculus]	304.24	0.95	-0.05	0.24	0.41	1.02	0.12	0.98	0.12	
		NADH dehydrogenase (ubiquinone) Fe-S protein 8 [Mus	232.04	0.95	-0.05	0.07	0.07	1.03	0.04	0.97	0.04	
		musculus]	927.53	0.95	-0.05	0.07	0.07	1.03	0.04	0.97	0.04	

NP_080886.1	2	NADH dehydrogenase 1 beta subcomplex 4 [Mus musculus]	302.68	0.94	-0.06	0.11	0.16	1.03	0.06	0.97	0.06
NP_062308.1	2	neuronal protein 15.6 [Mus musculus]	181.11	1.04	0.04	0.23	0.61	0.98	0.11	1.02	0.11
			1112.7								
NP_032736.2	2	nicotinamide nucleotide transhydrogenase [Mus musculus]	8	0.81	-0.21	0.08	0	1.11	0.04	0.89	0.04
			1035.0								
NP_035086.2	2	oxoglutarate dehydrogenase (lipoamide) [Mus musculus]	9	0.89	-0.12	0.12	0.04	1.06	0.06	0.94	0.06
NP_036151.1	2	peroxiredoxin 5 precursor [Mus musculus]	221.3	0.84	-0.18	0.27	0.1	1.09	0.13	0.91	0.13
PREDICTED: similar to acyl-CoA synthetase long-chain family member 1 isoform 2 [Mus musculus]											
XP_996295.1	2	PREDICTED: similar to acyl-CoA synthetase long-chain family member 1 isoform 2 [Mus musculus]	488.25	1.12	0.11	0.14	0.94	0.94	0.07	1.06	0.07
XP_996322.1	2	PREDICTED: similar to acyl-CoA synthetase long-chain family member 1 isoform 3 [Mus musculus]	221.57	1.06	0.06	0.15	0.72	0.97	0.07	1.03	0.07
XP_996347.1	2	PREDICTED: similar to acyl-CoA synthetase long-chain family member 1 isoform 4 [Mus musculus]	488.25	1.13	0.12	0.14	0.95	0.94	0.07	1.06	0.07
XP_996374.1	2	PREDICTED: similar to acyl-CoA synthetase long-chain family member 1 isoform 5 [Mus musculus]	488.25	1.13	0.12	0.12	0.96	0.94	0.06	1.06	0.06
PREDICTED: similar to ADP/ATP translocase 1 (Adenine nucleotide translocator 1) (ANT 1) (ADP,ATP carrier protein 1) (Solute carrier family 25 member 4) (ADP,ATP carrier protein, heart/skeletal muscle isoform T1) (mANC1) isoform											
XP_996193.1	2	2 [Mus musculus]	1498.8	0.99	-0.01	0.04	0.28	1.01	0.02	0.99	0.02
			5								
PREDICTED: similar to ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (Solute carrier family 25 member 5) [Mus musculus]											
XP_484885.2	2	PREDICTED: similar to ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (Solute carrier family 25 member 5) isoform 1 [Mus	573.17	1.03	0.03	0.09	0.71	0.99	0.05	1.01	0.05
XP_485652.2	2	PREDICTED: similar to ADP/ATP translocase 2 (Adenine nucleotide translocator 2) (ANT 2) (ADP,ATP carrier protein 2) (Solute carrier family 25 member 5) isoform 1 [Mus	520.89	0.97	-0.03	0.06	0.21	1.01	0.03	0.99	0.03
PREDICTED: similar to Aspartate aminotransferase, mitochondrial precursor (Transaminase A) (Glutamate											
XP_909149.1	2	musculus]	573.17	1.02	0.02	0.1	0.69	0.99	0.05	1.01	0.05
PREDICTED: similar to oxaloacetate transaminase 2) [Mus musculus]											
XP_923115.2	2	1038.7	1.07	0.07	0.06	0.99	0.96	0.03	1.04	0.03	
		6									
PREDICTED: similar to ATP synthase B chain,											
XP_995005.1	2	mitochondrial precursor [Mus musculus]	268.07	0.98	-0.02	0.1	0.33	1.01	0.05	0.99	0.05
PREDICTED: similar to ATP synthase B chain,											
XP_997887.1	2	mitochondrial precursor [Mus musculus]	268.07	0.98	-0.02	0.09	0.38	1.01	0.05	0.99	0.05
PREDICTED: similar to ATP synthase coupling factor 6, mitochondrial precursor (ATPase subunit F6) [Mus											
XP_980826.1	2	musculus]	270.62	0.99	-0.01	0.11	0.39	1	0.06	1	0.06
PREDICTED: similar to ATP synthase gamma chain, mitochondrial precursor [Mus musculus]											
XP_987162.1	2	591.93	0.93	-0.07	0.09	0.06	1.04	0.04	0.96	0.04	
PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit d [Mus musculus]											
XP_918900.1	2	356.6	1.03	0.03	0.13	0.69	0.98	0.07	1.02	0.07	
PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit d [Mus musculus]											
XP_891471.1	2	356.6	1.07	0.07	0.13	0.83	0.97	0.07	1.03	0.07	
PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit f, isoform 2 [Mus											
XP_999581.1	2	musculus]	131.66	0.93	-0.07	0.21	0.16	1.04	0.1	0.96	0.1

		PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit f, isoform 2 [Mus musculus]										
XP_895476.1	2	PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit [Mus musculus]	131.66	0.95	-0.05	0.19	0.27	1.02	0.09	0.98	0.09	
XP_998898.1	2	PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit [Mus musculus]	398.66	0.94	-0.06	0.07	0.06	1.03	0.03	0.97	0.03	
XP_484160.1	2	PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit [Mus musculus]	398.66	0.95	-0.05	0.06	0.06	1.03	0.03	0.97	0.03	
XP_911178.1	2	PREDICTED: similar to ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit [Mus musculus]	398.66	0.95	-0.05	0.07	0.1	1.02	0.03	0.98	0.03	
XP_994933.1	2	PREDICTED: similar to CG5903-PA [Mus musculus]	145.05	0.87	-0.14	0.28	0.17	1.07	0.14	0.93	0.14	
XP_920836.1	2	PREDICTED: similar to cytochrome c oxidase, subunit VIc [Mus musculus]	176.99	0.98	-0.02	0.1	0.36	1.01	0.05	0.99	0.05	
XP_999093.1	2	PREDICTED: similar to cytochrome c oxidase, subunit VIc [Mus musculus]	176.99	0.98	-0.02	0.11	0.36	1.01	0.06	0.99	0.06	
XP_988746.1	2	PREDICTED: similar to Cytochrome c, somatic [Mus musculus]	176.99	0.99	-0.01	0.13	0.46	1	0.06	1	0.06	
XP_996799.1	2	PREDICTED: similar to Cytochrome c, somatic [Mus musculus]	318.58	1.14	0.13	0.14	0.95	0.93	0.07	1.07	0.07	
XP_987982.1	2	PREDICTED: similar to Cytochrome c, somatic isoform 1 [Mus musculus]	318.58	1.15	0.14	0.14	0.97	0.93	0.07	1.07	0.07	
XP_980234.1	2	PREDICTED: similar to Cytochrome c, somatic isoform 2 [Mus musculus]	318.58	1.15	0.14	0.15	0.97	0.93	0.07	1.07	0.07	
XP_980266.1	2	PREDICTED: similar to Electron transfer flavoprotein beta-subunit (Beta-ETF) [Mus musculus]	318.58	1.15	0.14	0.11	0.98	0.93	0.06	1.07	0.06	
XP_914990.2	2	PREDICTED: similar to electron transferring flavoprotein, beta polypeptide [Mus musculus]	460.58	1.06	0.06	0.13	0.81	0.97	0.07	1.03	0.07	
XP_00100311.8.1	2	PREDICTED: similar to hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit [Mus musculus]	622.18	1.01	0.01	0.1	0.52	1	0.05	1	0.05	
XP_930842.1	2	PREDICTED: similar to NADH dehydrogenase (ubiquinone) [Mus musculus]	1046.44	1.34	0.29	0.06	1	0.86	0.03	1.14	0.03	
XP_987986.1	2	PREDICTED: similar to 1 alpha subcomplex, 9 [Mus musculus]	240.82	1.03	0.03	0.21	0.58	0.98	0.1	1.02	0.1	
XP_990709.1	2	PREDICTED: similar to 1, subcomplex unknown, 2 [Mus musculus]	255.3	0.89	-0.12	0.14	0.07	1.06	0.07	0.94	0.07	
XP_894449.1	2	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 3 [Mus musculus]	537.67	0.9	-0.1	0.11	0.05	1.05	0.05	0.95	0.05	
XP_894909.1	2	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 6 [Mus musculus]	278.57	0.81	-0.21	0.15	0	1.1	0.07	0.9	0.07	
XP_909811.1	2	PREDICTED: similar to NADH dehydrogenase (ubiquinone) Fe-S protein 6 [Mus musculus]	113.76	0.98	-0.02	0.3	0.43	1.01	0.15	0.99	0.15	
XP_987021.1	2	PREDICTED: similar to NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor isoform 1 [Mus musculus]	514.82	1.09	0.09	0.09	0.94	0.96	0.05	1.04	0.05	
XP_987060.1	2	PREDICTED: similar to NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor isoform 2 [Mus musculus]	574.55	1.07	0.07	0.09	0.93	0.97	0.05	1.03	0.05	
XP_922851.1	2	PREDICTED: similar to NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial precursor isoform 3 [Mus musculus]	574.55	1.06	0.06	0.1	0.94	0.97	0.05	1.03	0.05	

XP_978921.1	2	musculus] PREDICTED: similar to prohibitin [Mus musculus]	646.12		0.93	-0.07	0.1	0.11	1.04	0.05	0.96	0.05	
XP_979065.1	2	PREDICTED: similar to prohibitin [Mus musculus]	646.12		0.93	-0.07	0.11	0.18	1.04	0.05	0.96	0.05	
XP_983153.1	2	PREDICTED: similar to prohibitin [Mus musculus] PREDICTED: similar to pyruvate dehydrogenase	646.12		0.93	-0.07	0.11	0.12	1.04	0.05	0.96	0.05	
XP_981174.1	2	(lipoamide) beta [Mus musculus] PREDICTED: similar to solute carrier family 25, member 4	377.78		0.9	-0.11	0.14	0.07	1.06	0.07	0.94	0.07	
XP_996141.1	2	isoform 1 [Mus musculus]	1295.9		0.98	-0.02	0.04	0.25	1.01	0.02	0.99	0.02	
		PREDICTED: similar to Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) [Mus musculus]	275.35	Memb ne-WT- 2007091 3	913	913	913	913	913	Memb ne-WT- 2007091 3	913	913	
XP_896808.2	2	PREDICTED: similar to Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) [Mus musculus]	275.35	Memb ne-WT- 2007091 3	913	913	913	913	913	Memb ne-WT- 2007091 3	913	913	
XP_998318.1	2	[Mus musculus] PREDICTED: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4 isoform 1 [Mus	258.3		913	913	913	913	913				
XP_134169.3	2	musculus] PREDICTED: solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4 isoform 2 [Mus	1498.8	5	0.99	-0.01	0.04	0.32	1.01	0.02	0.99	0.02	
XP_902025.1	2	musculus] prohibitin [Mus musculus]	1295.9		0.97	-0.03	0.05	0.15	1.01	0.02	0.99	0.02	
NP_032857.1	2	646.12		0.92	-0.08	0.12	0.1	1.04	0.06	0.96	0.06		
NP_077183.1	2	pyruvate dehydrogenase (lipoamide) beta [Mus musculus] solute carrier family 25 (mitochondrial carrier oxoglutarate	400.22		0.89	-0.12	0.12	0.01	1.06	0.06	0.94	0.06	
NP_077173.1	2	carrier), member 11 [Mus musculus] solute carrier family 25 (mitochondrial carrier, adenine	419.78		0.96	-0.04	0.12	0.27	1.02	0.06	0.98	0.06	
NP_056644.1	2	nucleotide translocator), member 13 [Mus musculus] solute carrier family 25 (mitochondrial carrier, Aralar),	427.52		0.86	-0.15	0.14	0.02	1.07	0.07	0.93	0.07	
NP_766024.1	2	member 12 [Mus musculus] solute carrier family 25 (mitochondrial carrier, phosphate	666.47		0.96	-0.04	0.15	0.28	1.02	0.07	0.98	0.07	
NP_598429.1	2	carrier), member 3 [Mus musculus] solute carrier family 25 (mitochondrial carrier; adenine	635.68		0.93	-0.07	0.06	0.02	1.04	0.03	0.96	0.03	
NP_848473.1	2	nucleotide translocator), member 31 [Mus musculus]	292.91		0.93	-0.07	0.17	0.22	1.03	0.09	0.97	0.09	
NP_031477.1	2	solute carrier family 25, member 5 [Mus musculus]	1141.9		0.95	-0.05	0.05	0.03	1.02	0.03	0.98	0.03	
			1508.4										
NP_075770.1	2	succinate dehydrogenase Fp subunit [Mus musculus] succinate-CoA ligase, GDP-forming, alpha subunit [Mus	5		0.96	-0.04	0.07	0.1	1.02	0.03	0.98	0.03	
NP_063932.1	2	musculus] succinate-Coenzyme A ligase, ADP-forming, beta subunit	334.92		0.92	-0.08	0.21	0.2	1.04	0.1	0.96	0.1	
NP_035636.1	2	[Mus musculus] Tu translation elongation factor, mitochondrial [Mus	178.01		0.87	-0.14	0.2	0.12	1.07	0.1	0.93	0.1	
NP_766333.1	2	musculus] ubiquinol cytochrome c reductase core protein 2 [Mus	434.84		0.83	-0.19	0.18	0.02	1.1	0.09	0.9	0.09	
NP_080175.1	2	musculus] ubiquinol-cytochrome c reductase binding protein [Mus	1503.3		1.06	0.06	0.04	1	0.97	0.02	1.03	0.02	
NP_080495.1	2	musculus] ubiquinol-cytochrome c reductase core protein 1 [Mus	368.49		0.98	-0.02	0.13	0.38	1.01	0.06	0.99	0.06	
NP_079683.2	2		1776.7		1.01	0.01	0.06	0.56	1	0.03	1	0.03	

		musculus] ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1 [Mus musculus] upregulated during skeletal muscle growth 5 [Mus	9								
NP_079986.1	2		882.78	1	0	0.06	0.39	1	0.03	1	0.03
NP_075700.2	2	musculus]	239.24 1061.6	1.03	0.03	0.15	0.73	0.98	0.07	1.02	0.07
NP_035824.1	2	voltage-dependent anion channel 1 [Mus musculus]	9	1.05	0.05	0.09	0.85	0.97	0.04	1.03	0.04
NP_035825.1	2	voltage-dependent anion channel 2 [Mus musculus]	719.86	1	0	0.1	0.46	1	0.05	1	0.05
NP_035826.1	2	voltage-dependent anion channel 3 [Mus musculus]	319.85	1.06	0.06	0.12	0.84	0.97	0.06	1.03	0.06

Supplemental Table S2B. Proteomic Analysis of all Mitochondrial Matrix Proteins from CIRKO Mice.

Accession	OK	Description	Score	Unique	Matrix -WT- 20070 913:M atrix- TG- 20070 913_ Ratio	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) Ratio	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) Ratio	Matrix -WT- 20070 913:M atrix- TG- 20070 913_L og(e) Ratio	Matrix -TG- 20070 913_R el Profile	Matrix- TG- 20070 913_R el Profile	Matrix- WT- 20070 913_R el Profile	Matrix- WT- 20070 913_R el Profile
NP_080448.1	2	2,4-dienoyl CoA reductase 1, mitochondrial [Mus musculus] 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	470		0.94	-0.06	0.26	0.34	1.03	0.13	0.97	0.13
NP_663542.1	2	3-hydroxyisobutyryl-Coenzyme A hydrolase [Mus musculus]	398		0.93	-0.07	0.25	0.31	1.04	0.12	0.96	0.12
NP_666220.1	2	3-hydroxyisobutyryl-Coenzyme A hydrolase [Mus musculus]	241		0.95	-0.05	0.28	0.4	1.02	0.14	0.98	0.14
NP_077150.1	2	3-oxoacid CoA transferase 1 [Mus musculus]	1715		0.81	-0.21	0.1	0	1.1	0.05	0.9	0.05
NP_542142.1	2	acetyl-CoA synthetase 2-like [Mus musculus] acetyl-Coenzyme A acetyltransferase 1 precursor [Mus	507		0.95	-0.05	0.18	0.3	1.02	0.09	0.98	0.09
NP_659033.1	2	musculus] acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase) [Mus musculus]	1484		1.01	0.01	0.08	0.52	1	0.04	1	0.04
NP_803421.1	2	acetyl-Coenzyme A dehydrogenase, long-chain [Mus	2650		1.35	0.3	0.06	1	0.85	0.03	1.15	0.03
NP_031407.2	2	musculus] acetyl-Coenzyme A dehydrogenase, medium chain [Mus	1660		1.12	0.11	0.06	1	0.94	0.03	1.06	0.03
NP_031408.1	2	musculus] aconitase 2, mitochondrial [Mus musculus]	1342		1.31	0.27	0.06	1	0.87	0.03	1.13	0.03
NP_542364.1	2	acetyl-Coenzyme A dehydrogenase, short chain [Mus	4681		1.09	0.09	0.03	1	0.95	0.02	1.05	0.02
NP_031409.2	2	musculus] acetyl-Coenzyme A dehydrogenase, very long chain [Mus	942		1.4	0.34	0.15	1	0.83	0.07	1.17	0.07
NP_059062.1	2	musculus] adenylate kinase 3 [Mus musculus]	1180		0.66	-0.42	0.18	0	1.21	0.09	0.79	0.09
NP_067274.1	2	ALBU_BOVIN (P02769) Serum albumin precursor (Allergen	241		0.96	-0.04	0.39	0.47	1.02	0.19	0.98	0.19
NP_000101.0	2	Bos d 6) (BSA)	1496		0.72	-0.33	0.09	0	1.16	0.04	0.84	0.04
NP_033786.1	2	aldehyde dehydrogenase 2, mitochondrial [Mus musculus]	689		1	0	0.2	0.46	1	0.1	1	0.1
					Matrix- WT- 20070 913	Matrix- WT- 20070 913	Matrix- WT- 20070 913	Matrix- WT- 20070 913	Matrix- WT- 20070 913			
NP_780647.2	2	aldehyde dehydrogenase 4 family, member A1 [Mus	301	3	1.21	0.19	0.13	1	0.91	0.06	1.09	0.06
NP_598803.1	2	musculus] aldehyde dehydrogenase family 6, subfamily A1 [Mus	508		0.73	-0.31	0.21	0	1.15	0.11	0.85	0.11
NP_058054.2	2	musculus] ATP synthase, H ⁺ transporting mitochondrial F1 complex,	537		0.73	-0.31	0.21	0	1.15	0.11	0.85	0.11
NP_031531.1	2	beta subunit [Mus musculus] ATP synthase, H ⁺ transporting, mitochondrial F1 complex,	444		0.73	-0.31	0.24	0	1.15	0.12	0.85	0.12
NP_033867.1	2	alpha subunit, isoform 1 [Mus musculus] branched chain aminotransferase 2, mitochondrial [Mus	358		1.09	0.09	0.18	0.82	0.95	0.09	1.05	0.09
NP_083832.1	2	musculus] citrate lyase beta like [Mus musculus]	210		1.07	0.07	0.3	0.69	0.97	0.15	1.03	0.15

NP_080720.1	2	citrate synthase [Mus musculus]	996	1.11	0.1	0.06	1	0.95	0.03	1.05	0.03
NP_082221.1	2	citrate synthase-like protein [Mus musculus]	581	1.02	0.02	0.07	0.61	0.99	0.04	1.01	0.04
NP_036136.1	2	cytosolic acyl-CoA thioesterase 1 [Mus musculus]	282	1.15	0.14	0.2	0.9	0.93	0.1	1.07	0.1
NP_031887.2	2	dihydrolipoamide dehydrogenase [Mus musculus]	1109	0.99	-0.01	0.09	0.42	1	0.05	1	0.05
NP_663589.2	2	dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex) [Mus musculus]	785	1.03	0.03	0.13	0.66	0.98	0.06	1.02	0.06
NP_084501.1	2	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex) [Mus musculus]	677	1.07	0.07	0.14	0.84	0.97	0.07	1.03	0.07
NP_034153.2	2	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase) [Mus musculus]	787	1.3	0.26	0.1	1	0.87	0.05	1.13	0.05
NP_663590.2	2	electron transferring flavoprotein, alpha polypeptide [Mus musculus]	1585	1.12	0.11	0.08	1	0.94	0.04	1.06	0.04
NP_080971.2	2	electron transferring flavoprotein, beta polypeptide [Mus musculus]	1329	1.06	0.06	0.06	0.97	0.97	0.03	1.03	0.03
NP_000202.0	2	ENO1_YEAST (P00924) Enolase 1 (EC 4.2.1.11) (2-phosphoglycerate dehydratase 1) (2-phospho-D-glycerate hydro-lyase 1)	515	0.58	-0.54	0.14	0	1.26	0.06	0.74	0.06
NP_058052.1	2	enoyl Coenzyme A hydratase 1, peroxisomal [Mus musculus]	279	1.03	0.03	0.24	0.59	0.98	0.12	1.02	0.12
NP_444349.1	2	enoyl Coenzyme A hydratase, short chain, 1, mitochondrial [Mus musculus]	726	1.14	0.13	0.11	0.99	0.94	0.06	1.06	0.06
NP_613067.1	2	es1 protein [Mus musculus]	615	0.97	-0.03	0.1	0.24	1.02	0.05	0.98	0.05
NP_034339.1	2	fumarate hydratase 1 [Mus musculus]	1166	1.09	0.09	0.1	0.97	0.95	0.05	1.05	0.05
NP_032159.1	2	glutamate dehydrogenase 1 [Mus musculus]	444	0.93	-0.07	0.46	0.39	1.03	0.22	0.97	0.22
NP_034455.1	2	glutamate oxaloacetate transaminase 2, mitochondrial [Mus musculus]	1385	1.14	0.13	0.07	1	0.93	0.03	1.07	0.03
NP_032329.1	2	heat shock protein 1 (chaperonin 10) [Mus musculus]	381	0.93	-0.07	0.15	0.14	1.04	0.07	0.96	0.07
NP_034607.2	2	heat shock protein 1 (chaperonin) [Mus musculus]	387	1.21	0.19	0.24	0.93	0.91	0.12	1.09	0.12
NP_034611.1	2	heat shock protein 9A [Mus musculus]	1171	1	0	0.09	0.53	1	0.05	1	0.05
NP_058043.3	2	hydroxyacyl-Coenzyme A dehydrogenase type II [Mus musculus]	313	1.06	0.06	0.26	0.68	0.97	0.13	1.03	0.13
NP_849209.1	2	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit [Mus musculus]	1483	1.04	0.04	0.11	0.81	0.98	0.05	1.02	0.05
NP_663533.1	2	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit [Mus musculus]	753	0.73	-0.31	0.21	0	1.15	0.1	0.85	0.1
NP_083949.2	2	inner membrane protein, mitochondrial [Mus musculus]	600	0.78	-0.25	0.18	0.01	1.13	0.09	0.87	0.09
NP_766599.1	2	isocitrate dehydrogenase 2 (NADP+), mitochondrial [Mus musculus]	2469	0.94	-0.06	0.05	0.03	1.03	0.03	0.97	0.03
NP_083849.1	2	isocitrate dehydrogenase 3 (NAD+) alpha [Mus musculus]	853	1.28	0.25	0.08	1	0.88	0.04	1.12	0.04
NP_062800.1	2	isovaleryl coenzyme A dehydrogenase [Mus musculus]	377	1.02	0.02	0.29	0.54	0.99	0.14	1.01	0.14
NP_032238.1	2	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain [Mus musculus]	549	1.43	0.36	0.11	1	0.82	0.05	1.18	0.05
NP_032643.2	2	malate dehydrogenase 2, NAD (mitochondrial) [Mus musculus]	2730	0.97	-0.03	0.04	0.09	1.02	0.02	0.98	0.02
NP_598949.2	2	mitochondrial acyl-CoA thioesterase 1 [Mus musculus]	397	1.16	0.15	0.21	0.91	0.93	0.1	1.07	0.1
NP_035086.2	2	oxoglutarate dehydrogenase (lipoyamide) [Mus musculus]	1687	1.3	0.26	0.09	1	0.87	0.04	1.13	0.04
NP_031478.1	2	peroxiredoxin 3 [Mus musculus]	473	1.04	0.04	0.14	0.8	0.98	0.07	1.02	0.07
NP_036151.1	2	peroxiredoxin 5 precursor [Mus musculus]	974	0.98	-0.02	0.1	0.34	1.01	0.05	0.99	0.05

XP_143732.3	2	PREDICTED: histidine triad nucleotide binding protein 2 [Mus musculus] PREDICTED: similar to 10 kDa heat shock protein, mitochondrial (Hsp10) (10 kDa chaperonin) (CPN10) [Mus musculus]	171	1.02	0.02	0.31	0.52	0.99	0.16	1.01	0.16
XP_898273.1	2	PREDICTED: similar to 10 kDa heat shock protein, mitochondrial (Hsp10) (10 kDa chaperonin) (CPN10) [Mus musculus]	352	0.9	-0.11	0.16	0.12	1.05	0.08	0.95	0.08
XP_995914.1	2	PREDICTED: similar to 10 kDa heat shock protein, mitochondrial (Hsp10) (10 kDa chaperonin) (CPN10) [Mus musculus]	352	0.9	-0.1	0.16	0.13	1.05	0.08	0.95	0.08
XP_916456.1	2	PREDICTED: similar to 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	352	0.91	-0.09	0.16	0.19	1.05	0.08	0.95	0.08
XP_989864.1	2	PREDICTED: similar to 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	270	0.97	-0.03	0.37	0.43	1.01	0.18	0.99	0.18
XP_996439.1	2	PREDICTED: similar to 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	198	0.98	-0.02	0.36	0.46	1.01	0.18	0.99	0.18
XP_916687.1	2	PREDICTED: similar to 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor [Mus musculus]	261	1.03	0.03	0.38	0.56	0.99	0.19	1.01	0.19
XP_484008.1	2	PREDICTED: similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (HSP-65) [Mus musculus]	392	1.2	0.18	0.26	0.93	0.91	0.13	1.09	0.13
XP_981087.1	2	PREDICTED: similar to 60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (Heat shock protein 60) (HSP-60) (Mitochondrial matrix protein P1) (HSP-65) [Mus musculus]	392	1.25	0.22	0.21	0.97	0.89	0.1	1.11	0.1
XP_980447.1	2	PREDICTED: similar to Acyl-coenzyme A thioesterase 1 (Acyl-CoA thioesterase 1) (Inducible cytosolic acyl-coenzyme A thioester hydrolase) (Long chain acyl-CoA thioester hydrolase) (Long chain acyl-CoA hydrolase) (CTE-I) [Mus musculus]	286	1.12	0.11	0.19	0.83	0.94	0.1	1.06	0.1
XP_923115.2	2	PREDICTED: similar to Aspartate aminotransferase, mitochondrial precursor (Transaminase A) (Glutamate oxaloacetate transaminase 2) [Mus musculus]	1351	1.14	0.13	0.09	1	0.93	0.04	1.07	0.04
XP_995088.1	2	PREDICTED: similar to coiled-coil-helix-coiled-coil-helix domain containing 3 isoform 2 [Mus musculus]	246	0.78	-0.25	0.23	0	1.12	0.12	0.88	0.12
XP_914990.2	2	PREDICTED: similar to Electron transfer flavoprotein beta-subunit (Beta-ETF) [Mus musculus]	930	1.06	0.06	0.09	0.86	0.97	0.04	1.03	0.04
XP_001003118.1	2	PREDICTED: similar to electron transferring flavoprotein, beta polypeptide [Mus musculus]	1000	1.05	0.05	0.06	0.94	0.97	0.03	1.03	0.03
XP_992848.1	2	PREDICTED: similar to Glutamate dehydrogenase 1, mitochondrial precursor (GDH) isoform 2 [Mus musculus]	404	1.12	0.11	0.38	0.78	0.95	0.18	1.05	0.18
XP_992813.1	2	PREDICTED: similar to Glutamate dehydrogenase 1, mitochondrial precursor (GDH) isoform 3 [Mus musculus]	320	1.13	0.12	0.41	0.69	0.94	0.2	1.06	0.2
XP_909681.2	2	PREDICTED: similar to Histidine triad nucleotide-binding protein 2 (HINT-2) (HINT-3) [Mus musculus]	171	0.98	-0.02	0.24	0.48	1.01	0.12	0.99	0.12
XP_978522.1	2	PREDICTED: similar to Histidine triad nucleotide-binding protein 2 (HINT-2) (HINT-3) [Mus musculus]	171	1	0	0.33	0.48	1	0.16	1	0.16
XP_930842.1	2	PREDICTED: similar to hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase (trifunctional protein), beta subunit	704	0.75	-0.29	0.22	0	1.14	0.11	0.86	0.11

XP_00100178 0.1	2	[Mus musculus] PREDICTED: similar to Malate dehydrogenase, mitochondrial precursor [Mus musculus]	179		1.14	0.13	0.15	0.93	0.94	0.07	1.06	0.07
XP_982094.1	2	mitochondrial precursor [Mus musculus]	179		1.15	0.14	0.15	0.95	0.93	0.07	1.07	0.07
XP_989435.1	2	PREDICTED: similar to Malate dehydrogenase, mitochondrial precursor [Mus musculus]	179		1.15	0.14	0.13	0.99	0.93	0.07	1.07	0.07
XP_988293.1	2	PREDICTED: similar to oxoglutarate dehydrogenase-like [Mus musculus]	589	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_138959.5	2	PREDICTED: similar to oxoglutarate dehydrogenase-like isoform 1 [Mus musculus]	589	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_991516.1	2	PREDICTED: similar to oxoglutarate dehydrogenase-like isoform 1 [Mus musculus]	589	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_991547.1		PREDICTED: similar to oxoglutarate dehydrogenase-like isoform 2 [Mus musculus]	589	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_991579.1		PREDICTED: similar to oxoglutarate dehydrogenase-like isoform 3 [Mus musculus]	589	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_991618.1		PREDICTED: similar to oxoglutarate dehydrogenase-like isoform 4 [Mus musculus]	610	Matrix-WT- 2007091 3	Matrix- -WT- 20070 913							
XP_981174.1		PREDICTED: similar to pyruvate dehydrogenase (lipoamide) beta [Mus musculus]	616		1.09	0.09	0.12	0.93	0.95	0.06	1.05	0.06
XP_986100.1		PREDICTED: similar to Pyruvate dehydrogenase E1 component alpha subunit, somatic form, mitochondrial precursor (PDHE1-A type I) isoform 2 [Mus musculus]	654		1.32	0.28	0.12	1	0.86	0.06	1.14	0.06
XP_998318.1		PREDICTED: similar to Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) [Mus musculus]	406		0.97	-0.03	0.21	0.38	1.02	0.1	0.98	0.1
XP_896808.2		PREDICTED: similar to Stress-70 protein, mitochondrial precursor (75 kDa glucose-regulated protein) (GRP 75) (Peptide-binding protein 74) (PBP74) (P66 MOT) (Mortalin) [Mus musculus]	366		1.02	0.02	0.16	0.59	0.99	0.08	1.01	0.08
NP_036149.1		programmed cell death 8 [Mus musculus]	309		0.87	-0.14	0.27	0.14	1.07	0.13	0.93	0.13
NP_077183.1		pyruvate dehydrogenase (lipoamide) beta [Mus musculus]	847		1.16	0.15	0.12	1	0.93	0.06	1.07	0.06
NP_032836.1		pyruvate dehydrogenase E1 alpha 1 [Mus musculus]	654		1.34	0.29	0.11	1	0.86	0.05	1.14	0.05

			Matrix- WT- 2007091	Matrix -WT- 20070	Matrix -WT- 20070	Matrix -WT- 20070	Matrix -WT- 20070
NP_063932.1	succinate-CoA ligase, GDP-forming, alpha subunit [Mus musculus]	443	3	913	913	913	913
NP_035636.1	succinate-Coenzyme A ligase, ADP-forming, beta subunit [Mus musculus]	478		1.65	0.5	0.14	1
NP_038699.2	superoxide dismutase 2, mitochondrial [Mus musculus]	801		1.02	0.02	0.11	0.63
NP_080066.1	thioesterase superfamily member 2 [Mus musculus]	165		1.7	0.53	0.26	1
NP_766333.1	Tu translation elongation factor, mitochondrial [Mus musculus]	443		0.83	-0.19	0.24	0.09

SUPPLEMENTAL FIGURES AND LEGENDS

SUPPLEMENTAL FIGURE S1. Purity of mitochondrial subfractions.

- A)** Protein content of the matrix protein MnSOD and the inner mitochondrial membrane protein complex IV subunit 4 in whole mitochondrial fraction (WMF), mitochondrial matrix (MT), and mitochondrial membranes (MB).
- B)** Protein content of the outer mitochondrial membrane protein mitofusin 2 (Mfn2) in mitochondrial matrix and membrane fractions.

SUPPLEMENTAL FIGURE S2. Contractile parameters and MVO₂ under low and high calcium conditions.

- A)** Left ventricular developed pressure (LVDVP) at baseline (2 mmol/L extracellular calcium concentrations) and following calcium-induced inotropic stress (4 mmol/L extracellular calcium concentrations) in 8 week-old Langendorff-perfused CIRKO (black bars) and wild-type (open bars) hearts.
- B)** Same as **A** for rate pressure product (RPP)
- C)** Same as **A** for dP/dt_{min} and dP/dt_{max}
- D)** Same as **A** for myocardial oxygen consumption (MVO₂)

Data are means \pm SEM. * p < 0.05; ** p < 0.005 vs. equivalently treated wild-type controls. # p<0.05; ## p<0.005 vs. 2mM Ca²⁺

SUPPLEMENTAL FIGURE S3. Maximal electron transport chain capacity in isolated mitochondria from 24 week-old wild-type and CIRKO mice.

(A) and **(B)** respiration traces of isolated mitochondria (0.4 mg/ml) from hearts of 24 week-old wild-type and CIRKO mice respectively. Respirations were carried in the presence of pyruvate-malate. Uncoupling of the electron transport chain was achieved by addition of FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone).

(C) Summary of states of respiration as detailed above in wild-type ($n = 4$) and CIRKO ($n=4$).

SUPPLEMENTAL FIGURE S4. Age-dependent decline in mitochondrial enzyme activities.

A) Fold change in citrate synthase (CS) activity in 8 (five per group), 24 (four to five per group) and 54 (three per group) week-old CIRKO (black bars) and wild-type (white bars). Each value represents fold change in CS activity in CIRKO mice relative to wild-type mice, which is arbitrarily defined as 1. Statistics: Wild-type (WT) vs. CIRKO, $p < 0.04$ at 24 and 54-weeks.

B) Fold changes in Carnitine palmitoyl-transferase 1 (CPT1) activity in 8 (seven per group), 24 (four to six per group) and 54 (five to six per group) week-old CIRKO (black bars) and wild-type (white bars). Each value represents fold change in CPT1 activity in CIRKO mice relative to that in wild-type mice, which is arbitrarily defined as 1. Statistics: WT vs. CIRKO, $p < 0.01$ at 8 and 54-weeks.

C) Fold changes in 3-Hydroxyacyl-CoA dehydrogenase (HADH) activity in 8 (five per group), 24 (four to five per group) and 54 (three per group) week-old CIRKO (black bars) and wild-type (white bars). Each value represents fold change in HADH activity in

CIRKO mice relative to wild-type mice, which is arbitrarily defined as 1. Statistics: WT vs. CIRKO, p<0.05 at 8 and 54-weeks.

SUPPLEMENTAL FIGURE S5. Age-related changes in gene expression in wild-types (open circles) and CIRKO (filled circles), at 3, 8 and 24-weeks.

SUPPLEMENTAL FIGURE S6. PDHE1 α protein expression in 14 week-old male wild-type and CIRKO mice.

- A)** Upper panel represents phospho-PDHE1 α and lower panel is total PDHE1 α expression in whole heart homogenates of 6 wild-types and 4 CIRKO mice.
- B)** Densitometric analysis. Phosphorylation state was normalized to total PDHE1 α and data represent fold change versus WT considered as 1.

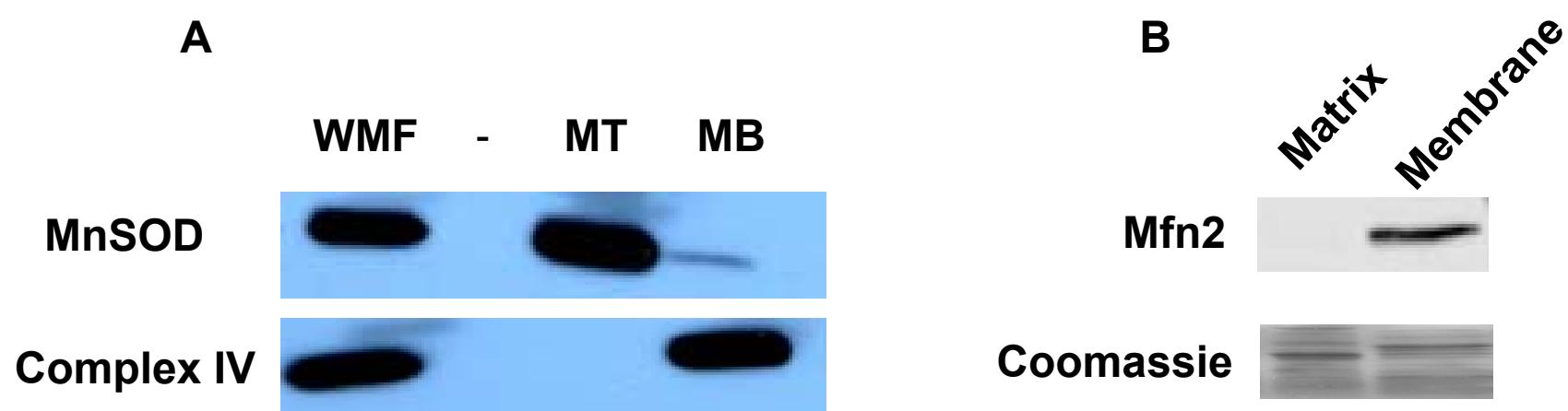
SUPPLEMENTAL FIGURE S7. IGF1-Receptor expression and insulin signaling in 8 week-old wild-type and CIRKO mice. WT and CIRKO hearts were perfused or not with 1 nM insulin.

- A)** *Upper panel*, IGF1-Receptor expression in WT and CIRKO whole heart homogenates. *Lower panel*, densitometric analysis. IGF1-R expression was normalized to Coomassie blue staining (CB). n=7-8 per group.
- B)** and **C)** Phospho-AKT-Ser⁴⁷³ and Phospho-GSK3 β -Ser⁹ immunoblotting and respective densitometric analysis. Phosphorylation state was normalized to total AKT or

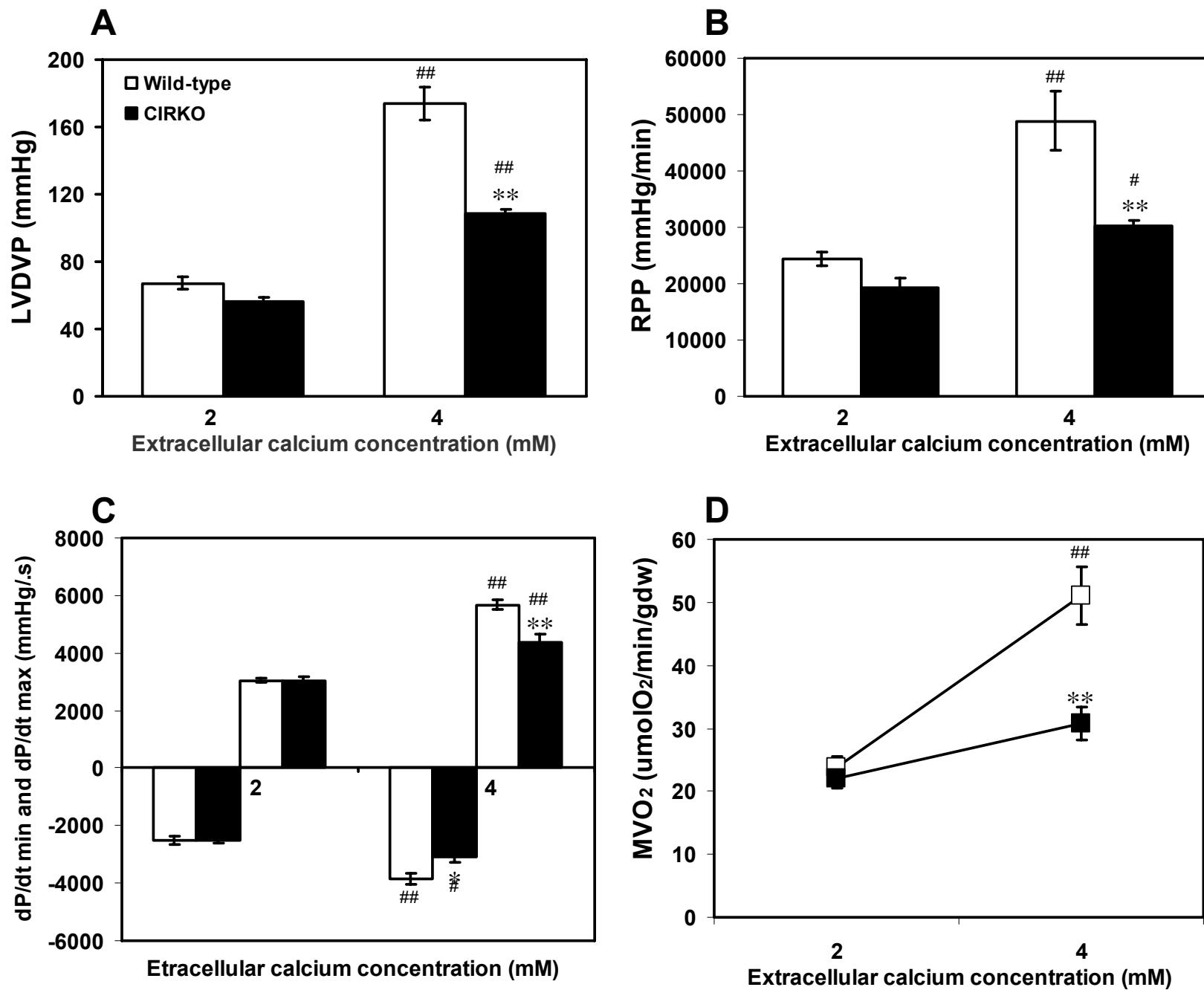
total GSK3 β expression, respectively. n=3-4 per group. #, * p<0.05 compared to WT or WT basal respectively.

SUPPLEMENTAL FIGURE S8. Palmitate oxidation (**A**), Oxygen consumption (**B**) and cardiac efficiency (**C**) in isolated-working hearts from 8 week-old CIRKO and wild-type mice. * p < 0.05; ** p < 0.005 vs. age-matched wild-type controls

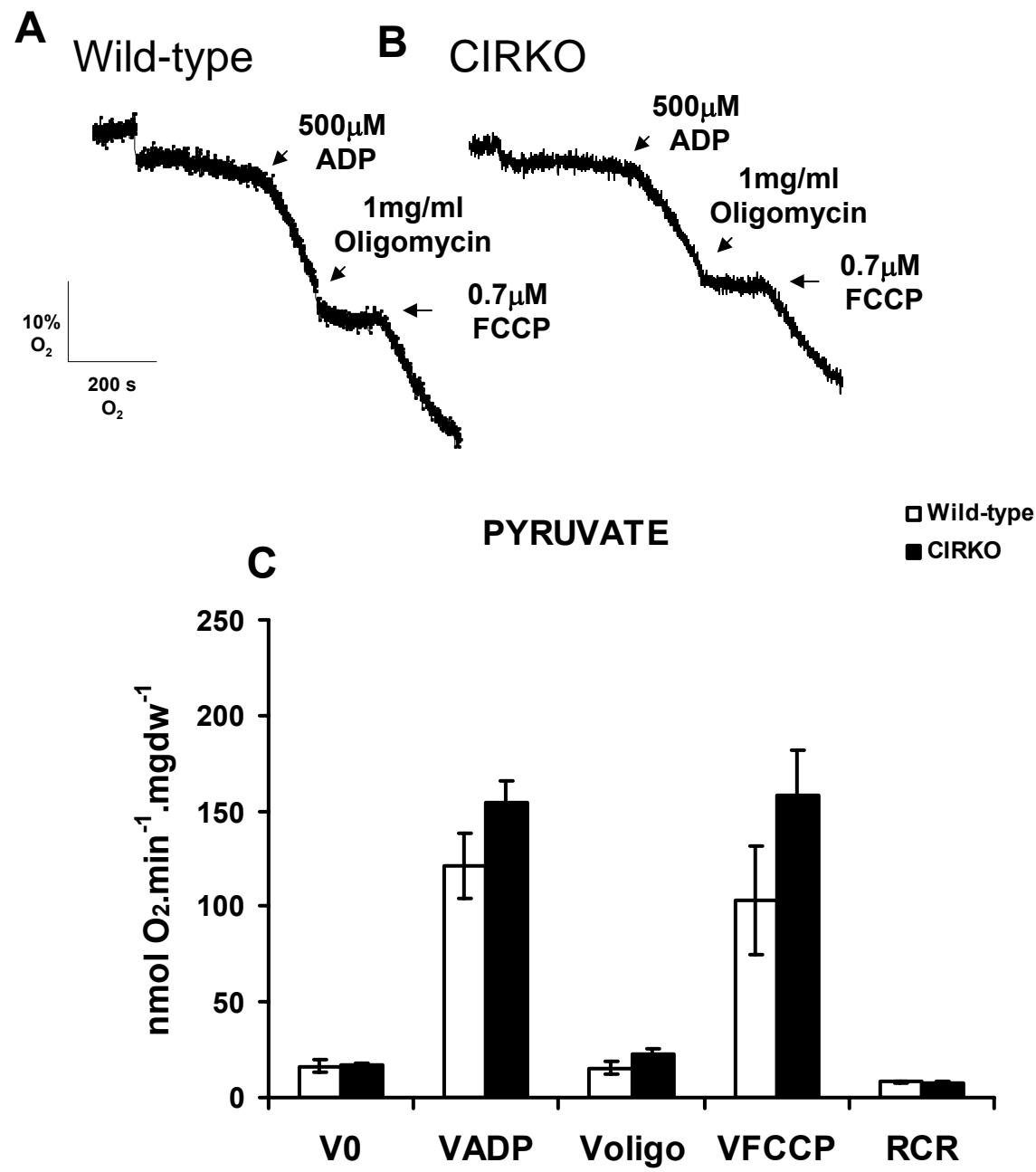
SUPPLEMENTAL FIGURE S1



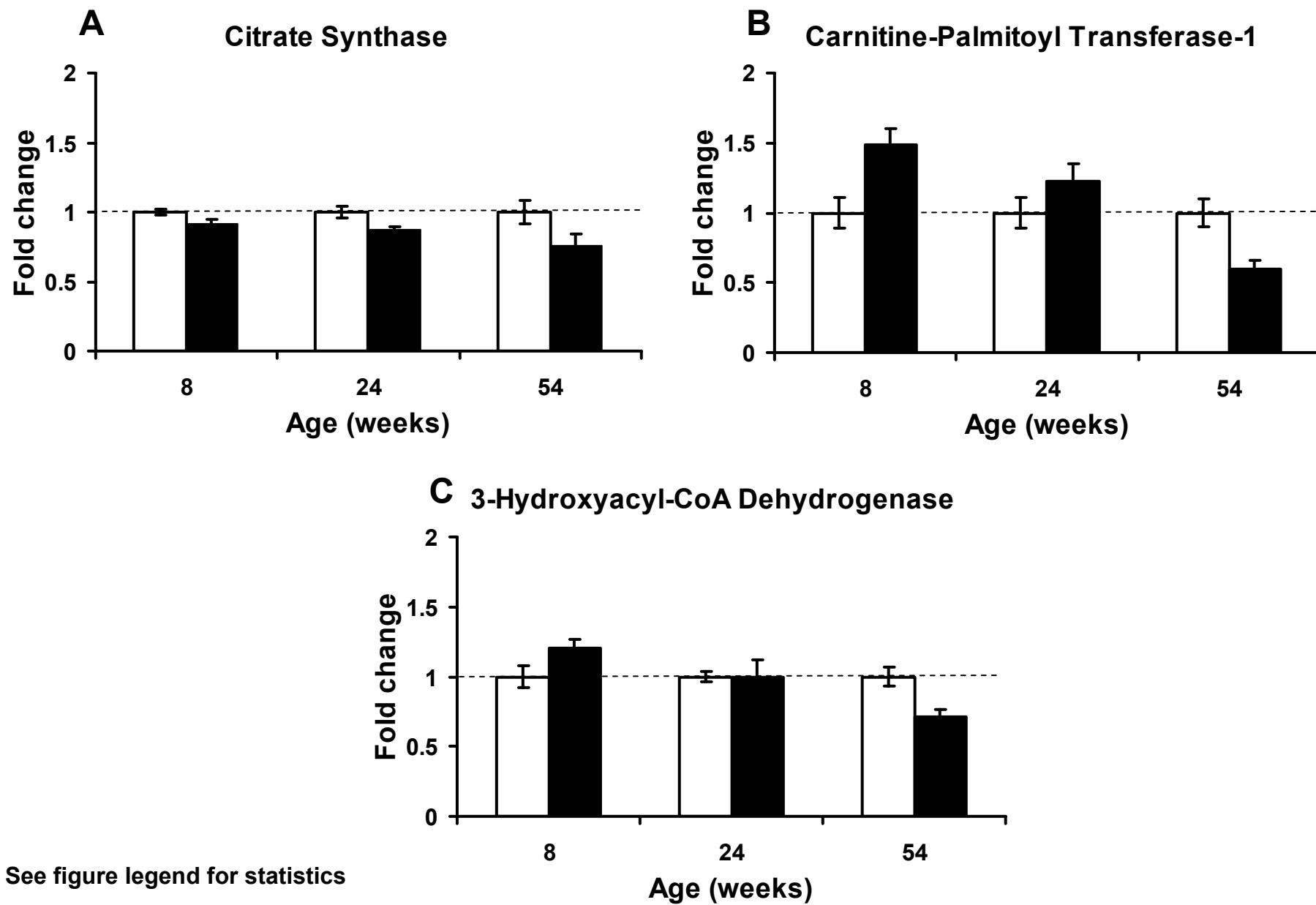
SUPPLEMENTAL FIGURE S2



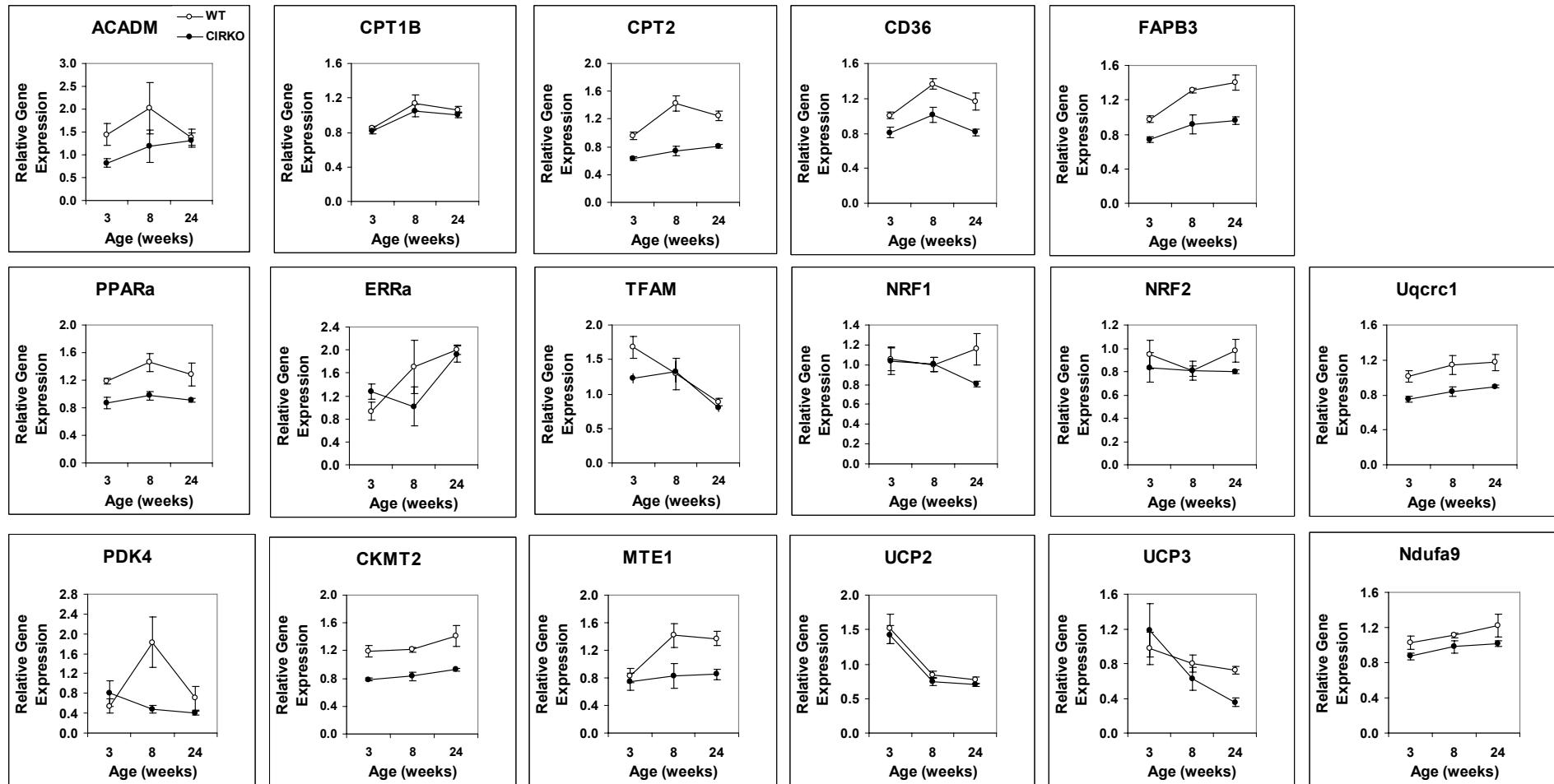
SUPPLEMENTAL FIGURE S3



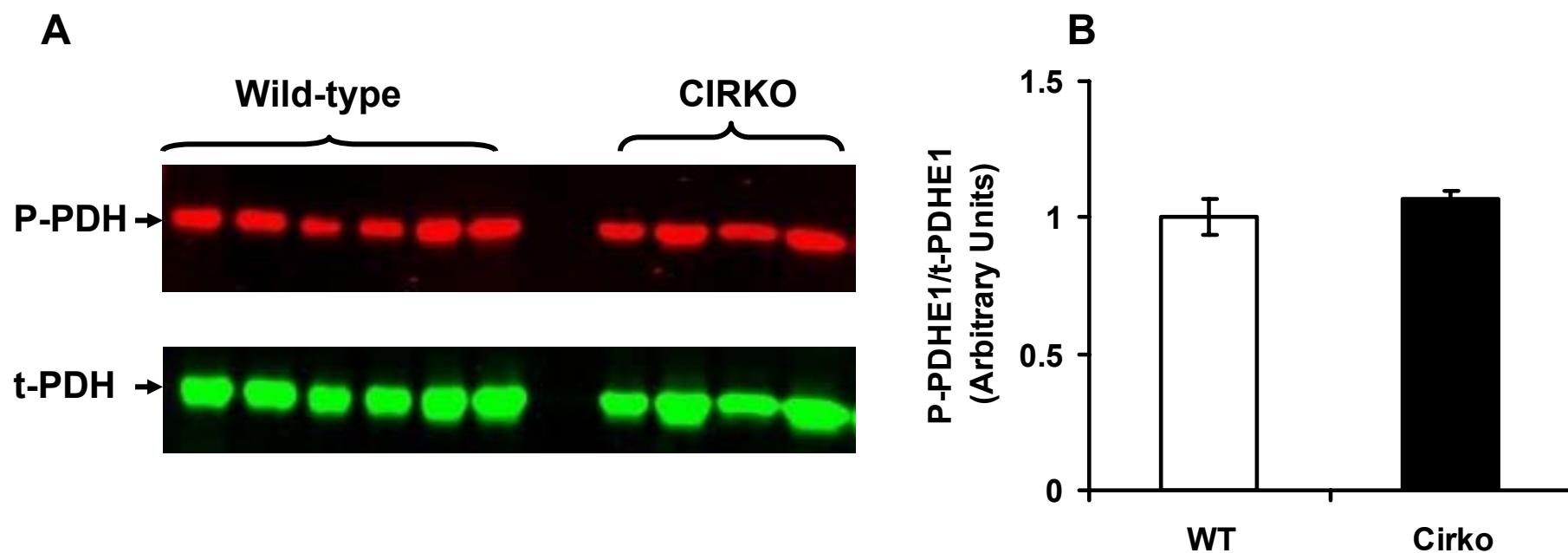
SUPPLEMENTAL FIGURE S4



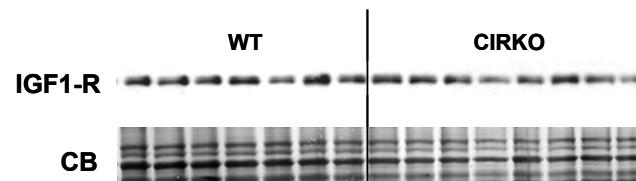
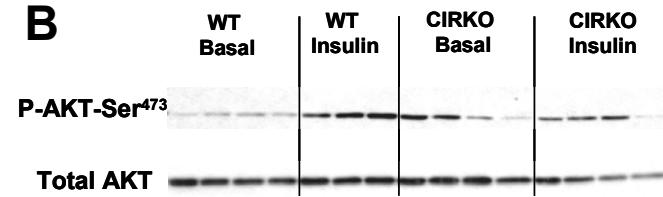
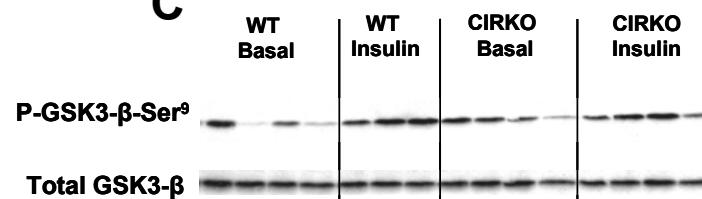
SUPPLEMENTAL FIGURE S5



SUPPLEMENTAL FIGURE S6



SUPPLEMENTAL FIGURE S7

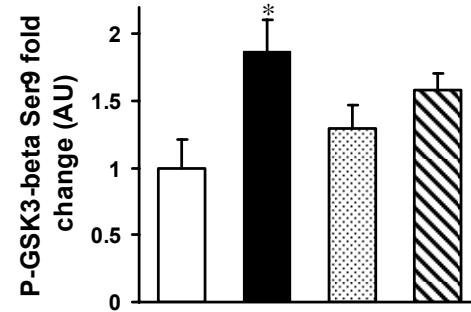
A**B****C**

□ WT Basal

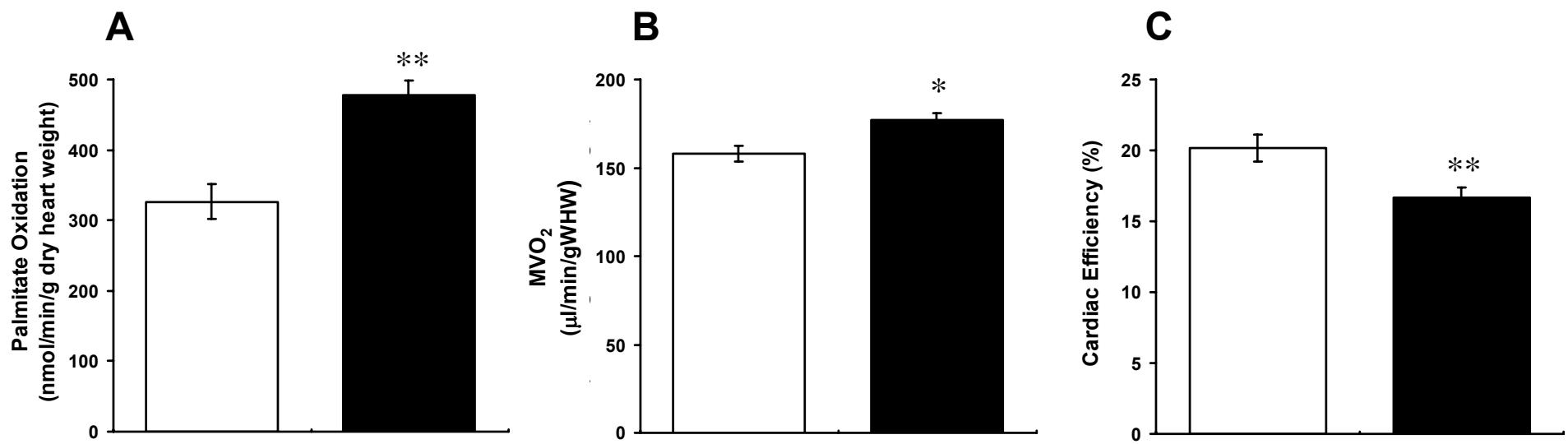
■ WT + Insulin 1 nM

▨ CIRKO Basal

▨ CIRKO + Insulin 1 nM



SUPPLEMENTAL FIGURE S8



REFERENCES

1. Zerbetto E, Vergani L, Dabbeni-Sala F. Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels. *Electrophoresis*. 1997;18:2059-2064.
2. Silva JC, Denny R, Dorschel CA, Gorenstein M, Kass IJ, Li GZ, McKenna T, Nold MJ, Richardson K, Young P, Geromanos S. Quantitative proteomic analysis by accurate mass retention time pairs. *Analytical chemistry*. 2005;77:2187-2200.
3. Boudina S, Sena S, O'Neill BT, Tathireddy P, Young ME, Abel ED. Reduced mitochondrial oxidative capacity and increased mitochondrial uncoupling impair myocardial energetics in obesity. *Circulation*. 2005;112:2686-2695.
4. Yan LJ, Levine RL, Sohal RS. Oxidative damage during aging targets mitochondrial aconitase. *Proc Natl Acad Sci U S A*. 1997;94:11168-11172.